Studies on Bio-fortification of Rice Bran Tocotrienol and Its Application for Nutraceutical Purpose

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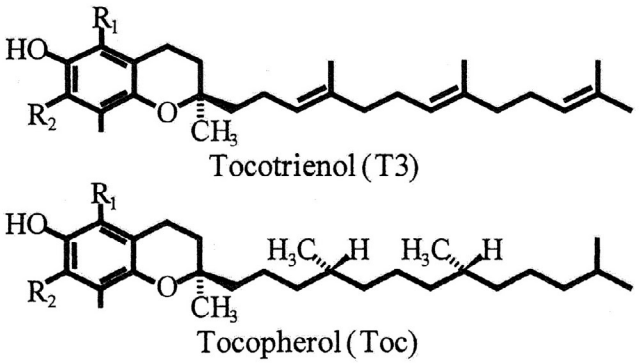
論 文 内 容 要 旨

Background

Rice bran has been known to contain some bio-active compounds such as tocotrienol (T3, unsaturated vitamin E) and tocopherol (Toc, saturated vitamin E) (**Fig. 1**). Structurally, T3 possesses an unsaturated isoprenoid tail with three double bonds, whereas Toc has a saturated phytyl side chain. In recent years, T3 has gained much attention for its several physiological properties that differ somewhat from those of Toc [1,2]. T3 shows better anti-oxidative, anti-hypercholesterolemic, anti-cancer, and neuroprotective activities than those of Toc. Moreover, in our laboratory, T3 has been demonstrated to suppress pathological angiogenesis, which is an important stage in progression of many severe disorders (i.e., diabetic retinopathy, rheumatoid arthritis, and cancers) [3,4]. These beneficial effects suggest T3 as a rice bran constituent with a wide variety of health benefits.

Despite the potential significance of T3, knowledge on how to apply rice bran T3 for health promotion has been poorly understood. Therefore, in this study, utilization and nutritional application of rice bran T3 has been investigated. The first part was to study on bio-fortification of rice bran T3 by screening T3-rich rice variety, improving rice bran T3 by cross-fertilization, and clarifying T3 biosynthesis of the progenies by using a quantitative trait locus (QTL) analysis. The second part of this study was aimed at utilization of rice bran T3 by understanding human daily intake of T3, and developing T3 products such as rice bran T3 concentrate and T3-fortified foods.



	R_1	R_2
α-	CH_3	CH_3
β-	CH_3	H
γ-	H	CH_3
δ-	H	H

Figure 1. Chemical structures of tocotrienol and tocopherol

Chapter 1. Bio-fortification of rice bran T3

1-1. Screening of T3-rich rice plant varieties

Introduction Since rices harvested from different areas vary in shape, texture, and flavor, it is possible for a variation of T3 in rice bran (T3-rich rice bran may be available). In this part of study, a rapid quantitation method for T3 and Toc was developed and used in screening of T3-rich rice varieties from various rice bran samples [5].

Methodology and results The extraction of T3 and Toc from rice bran was employed by one-step solvent extraction with 2-propanol, and the determination of T3 and Toc in the rice bran extract was performed by high-performance liquid chromatography (HPLC) with fluorescence detection using an Inersil SIL 100A-5 column (4.6 × 250 mm; GL Science) and a mobile phase of hexane/1,4-dioxane /2-propanol (1000/40/5, v/v/v). The one-step extraction method provided high recovery rate (>95%) of T3 isoforms, Toc isoforms and the internal standard 2,2,5,7,8-pentamethyl-6-hydroxychromane (PMC) (**Table 1**). The analysis time for the HPLC determination was within 15 min for each replication (**Fig. 2**). Using the developed methods, 251 kinds of rice bran samples collected around the world were determined for their T3 and Toc. As results, there was a wide variation of T3 contents in rice bran samples (350-1460 μg/g dry wt for the rice bran samples prepared in 2005). Some varieties such as Milyang23, Wataribune, and Padi Perek were found as T3-rich varieties (1460, 1345, and 1340 μg/g, respectively), whereas Koshihikari (a reference variety) did not contain much level of T3 (880 μg/g) (**Fig. 3**). The quantitative data from different year (2006) as well as from different transplanting times (**Fig. 4**) also revealed that Milyang23 was the best variety having high content of T3. Milyang23 was therefore chosen for cross-fertilization with Koshihikari for improving rice bran T3 in consequent experiment.

Table 1. Recovery rates of PMC, α-, β-, γ -, and δ- isoforms of T3 and Toc.

	Content (μg/g dry wt)								
	PMC	α-T3	β-T3	γ-T3	δ-T3	α-Toc	β-Toc	γ-Toc	δ-Toc
Cp	-	296.4	4.49	567	31.2	455.5	17.0	85.4	39.7
Ca	265.8±2.5	297.2±3.8	4.50±0.09	567±1.1	31.4±0.7	454.5±4.6	17.0±0.1	85.9±0.5	39.7±0.1
Cs	263.2±0.6	587.5±3.8	8.80±0.17	1121±1.9	62.6±0.4	900.7±10	33.8±0.3	169.3±0.5	79.1±0.2
Cs-Cp	263.2±0.6	291.1±2.8	4.30±0.01	554±0.1	31.4±0.1	445.3±0.8	16.8±0.1	83.9±0.4	39.4±0.2
R%	99.0±0.7	98.0±1.6	97.3±1.60	97.7±1.7	100.1±0.7	98.0±1.0	98.8±1.5	97.7±1.5	99.4±1.4

Value are expressed as mean±S.D., n = 3

Recovery rate was calculated by the equation: R% = [(Cs-Cp)/Ca] × 100. Where R (%) is the percent recovery of added vitamin E; Cs, the vitamin E content in spiked sample (Koshihikari); Cp, the vitamin E content in sample; and Ca, the vitamin E added.

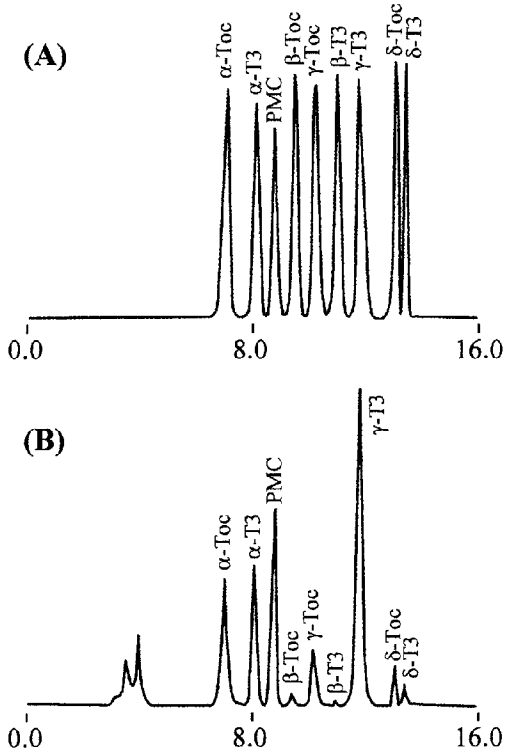


Figure 2. Typical HPLC chromatograms of standard vitamin E (A) and a rice bran extract (Koshihikari) (B). An Inersil SIL 100A-5 (4.6 × 250 mm) column was used with a mobile phase of hexane/1,4-dioxane/2-propanol (1000:40:5, v/v/v) at a flow rate of 1.0 mL/min. The column temperature was maintained at 35°C. The fluorescence detection was set as 294 nm for excitation and 326 nm for emission wavelengths.

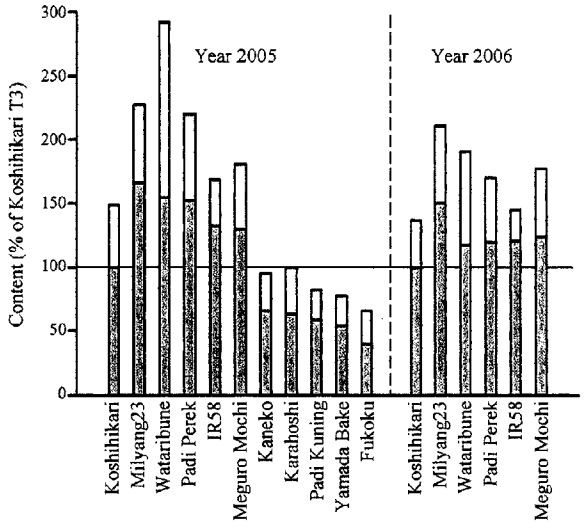


Figure 3. Total T3 (shown in gray) and Toc (shown in white) content of some rice varieties. The T3 of the reference Koshihikari was shown as 100%.

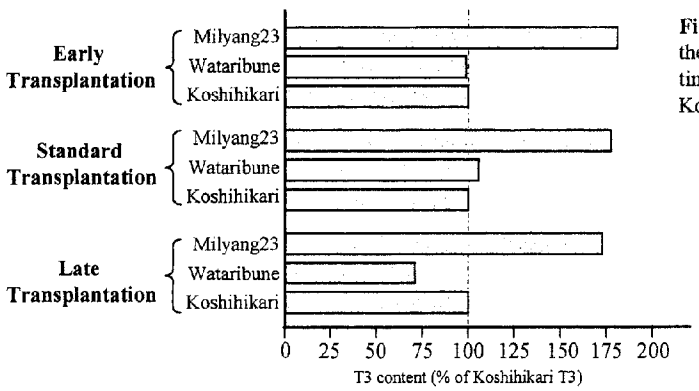


Figure 4. Total T3 content of some rice varieties when the rice plants were raised at the different transplanting times. T3 contents are presented as percentages to that of Koshihikari T3.

1-2. Cross-breeding of Koshihikari with the T3-rich rice variety and QTL analysis for understanding T3 biosynthesis

Introduction From the previous part, it was found that Koshihikari did not contained much content of T3, whereas Milyang23 was the cultivar with a reliable high content of T3. The findings suggest that cross-breeding of Koshihikari with Milyang23 would result a higher distribution of T3 content and some of their progenies would be expected to be rich in T3 content. Their F_2 progenies could be useful in clarifying T3 biosynthesis in rice plants by using a QTL analysis.

Methodology and results Milyang23 was cross-fertilized with Koshihikari and 133 F_2 individuals were obtained. Each F_2 individual was determined for T3 content, and a frequency distribution of T3 content (total T3 isoforms) of their F_2 was in the range of 60-200% Koshihikari T3 (**Fig. 5**). The average T3 content of the F_2 progenies was 135% Koshihikari T3, and there were 32 samples having T3 content more than that of Milyang23 (24% of the total F_2 populations). The different T3 levels in the F_2 would be an outcome from the variation of genetic traits of the both parental lineages. Consequently, a QTL analysis for high T3 biosynthesis was performed. A genetic linkage map was constructed using 173 simple sequence repeat (SSR) markers (**Fig. 6**) and a threshold LOD score more than 2.5 was used for QTL detection. As results, five putative QTLs responding T3 production were found on chromosome 1 (*qT3-1*), chromosome 6 (*qT3-6-1* and *qT3-6-2*), and chromosome 9 (*qT3-9-1* and *qT3-9-2*) (**Fig. 6, Table 2**). The major QTLs on the chromosome 6 (*qT3-6-1*; LOD=11.3 and *qT3-6-2*; LOD=10.2) were mapped near the SSR markers RM3827 and RM7434 (**Fig. 7**). This suggests that the QTLs on chromosome 6 have close relation to T3 biosynthesis, which is in accordance with other studies on biosynthesis of T3 in plants (e.g., isolation of cDNA of enzymes for vitamin E synthesis from chromosome 6 of rice plant) [6,7]. Therefore, QTLs located outside chromosome 6 would rather relate to biosynthesis of vitamin E precursors (i.e., acetyl CoA and geranylgeranyl diphosphate) or other supplementary events supporting biosynthesis of T3. By further cross-breeding of the F_2 individuals with high T3 content and genetic confirming of the chromosomal regions of the detected QTLs, T3 could be more synthesized and accumulated in rice bran.

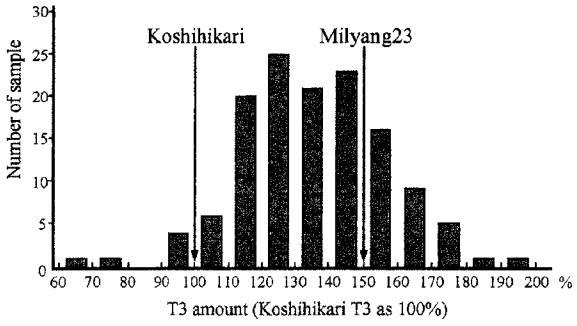


Figure 5. Frequency distribution of T3 content (total T3 isoforms) of the 133 F_2 progenies of Koshihikari and Milyang23. Values were expressed as %T3 compared with that of the parent Koshihikari.

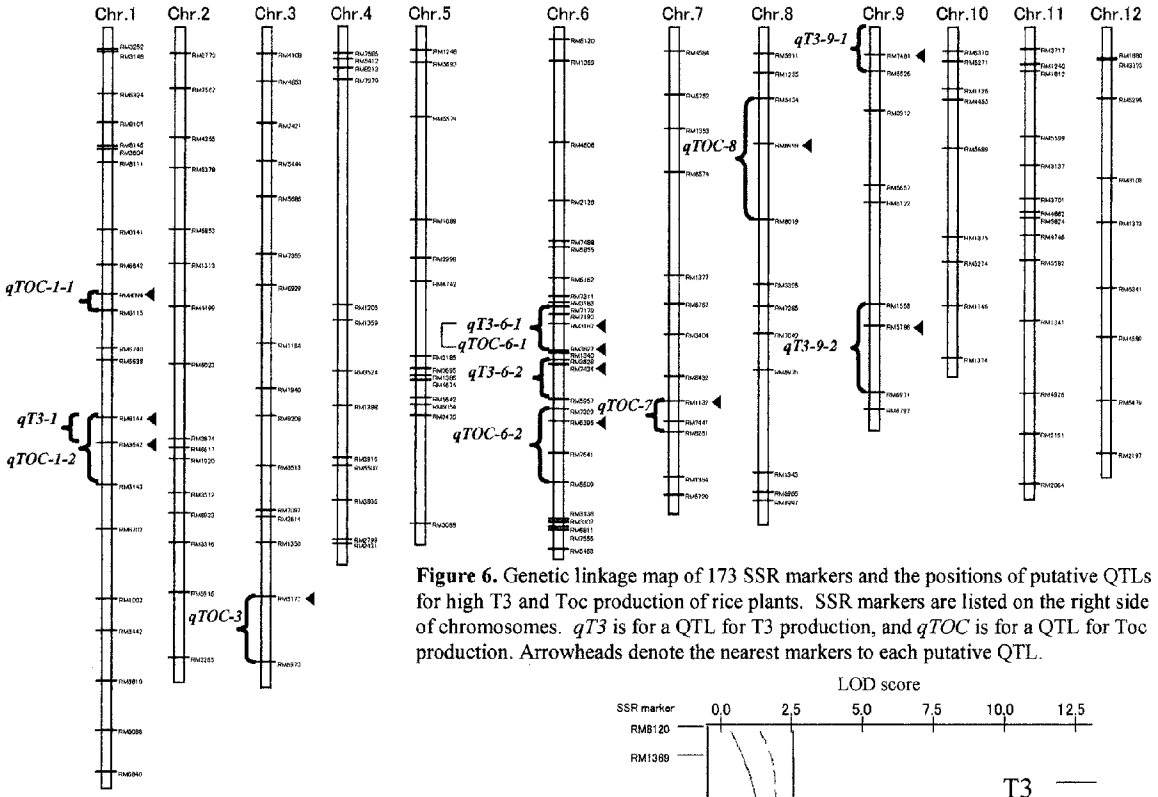


Figure 6. Genetic linkage map of 173 SSR markers and the positions of putative QTLs for high T3 and Toc production of rice plants. SSR markers are listed on the right side of chromosomes. *qT3* is for a QTL for T3 production, and *qTOC* is for a QTL for Toc production. Arrowheads denote the nearest markers to each putative QTL.

Table 2. Putative QTLs for the T3 and Toc contents in rice bran

Traits	QTL	NML	Chromosome	LOD
T3	*qT3-1*	**RM8144**	**1**	**2.7**
	qT3-6-1	**RM3827**	**6**	**11.3**
	qT3-6-2	**RM7434**	**6**	**10.2**
	qT3-9-1	**RM7481**	**9**	**3.9**
	qT3-9-2	**RM5786**	**9**	**4.2**
Toc	*qTOC-1-1*	**RM8094**	**1**	**2.5**
	qTOC-1-2	**RM3642**	**1**	**3.2**
	qTOC-3	**RM5172**	**3**	**2.5**
	qTOC-6-1	**RM3187**	**6**	**4.7**
	qTOC-6-2	**RM6395**	**6**	**4.4**
	qTOC-7	**RM1132**	**7**	**2.7**
	qTOC-8	**RM6999**	**8**	**2.6**

NML : Nearest marker locus to putative QTL.

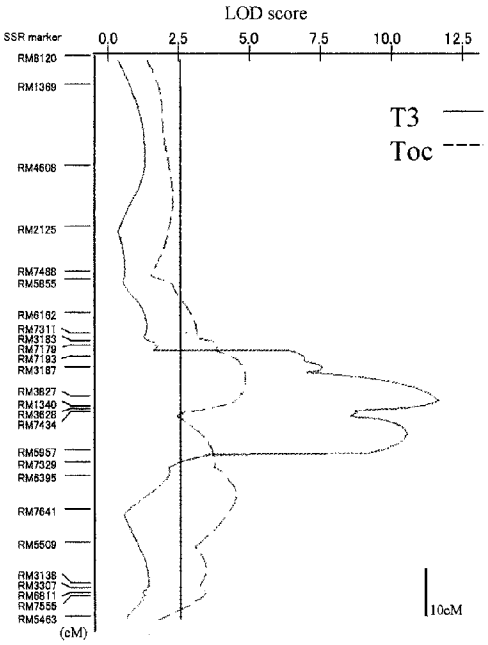


Figure 7. High resolution of LOD score plot on chromosome 6 for the QTLs for the occurrence of high T3 production in the F_2 of Koshihikari and Milyang23. The detection threshold was LOD = 2.50.

Chapter 2. Application of rice bran T3 for nutraceutical purpose

2-1. Estimation of human daily intake of T3

Introduction Due to potential health benefits of T3, it has been received much attention on its nutraceutical application. However, to date, human daily intake of T3 has been poorly understood.

Methodology and results To understand daily intake of T3, 242 food items and 64 processed meals were measured for their T3 content (**Fig. 8**). T3 contents in food items were non-detectable to 12 mg of T3/kg, and daily intake of T3 was estimated as 2.1 mg T3/day/person (sum of T3 intake of each food category (e.g., cereals, grains, and fruits)). Correspondingly, T3 contents in process meals were from non-detectable to 1.3 mg of T3/meal, and daily intake of T3 was estimated as 1.9 mg T3/day/person (3× sum of average T3 in rice, accompanying dish, and soup). Since daily intake of Toc is around 8-10 mg/day/person, the daily intake of T3 seems rather low and extra amount of T3 would be required for therapeutic aspect of T3.

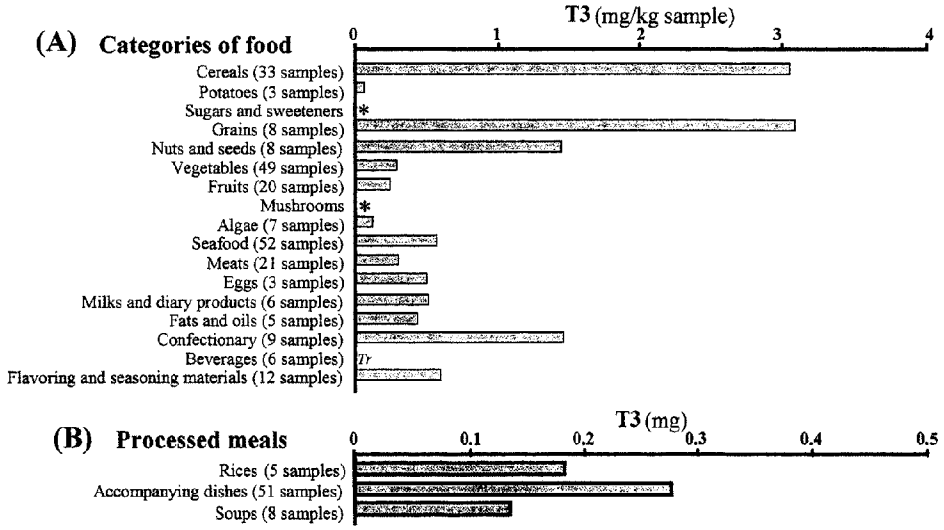


Figure 8. Average T3 contents of analyzed foods. (A) by mean of categories of food as classified by the Ministry of Health, Labor and Welfare. (B) by mean of processed meals, in which a meal consists of a dish of rice, a set of accompanying dish (2-3 sub-menus), and a small bowl of soup. *No quantitation determined in categories of sugars and sweeteners and of mushrooms because they have been reported no contents of Toc implying lack of T3.

2-2. Separation of rice bran T3 by simulated moving-bed chromatography

Introduction An industrial production of high purity T3 seems very difficult because of its structural similarity with Toc. Technologies (e.g., molecular distillation) have been used in separation of T3, but with co-existence of Toc [8]. A new simulated moving-bed (SMB) system is a counter current chromatography capable to separate three zones of mixtures, and has been used in purification of pharmaceuticals [9]. In this study, the new SMB chromatography was used as a tool for separation of rice bran T3 (**Fig. 9**).

Methodology and results To select proper types of adsorbent and eluent, normal-phase (NP) and reverse-phase (RP) HPLC systems with various eluents (mixtures of ethanol/water/n-hexane/acetone) were tested. As results, the RP ODS system with an eluent of ethanol/water allowed phase separation between T3 and Toc, while the NP silica system did not (**Fig. 10**). Therefore, the RP system was chosen for SMB operation. Consequently, to acquire parameter for the SMB separation, a rice bran concentrate (containing 20% T3 and 20% Toc, Sanwa-Yushi Co., Ltd, Tendo, Japan) was eluted through four connected ODS columns (a single-pass test), and by computer simulation the elution profiles of each constituent of the rice bran concentrate were estimated (**Fig. 11**). The assignment of zone separation for the SMB system was as *A* fraction (low affinity impurities), *B* fraction (T3 fraction), and *C* fraction (Toc fraction and high affinity impurities) (**Fig. 11**). The rice bran concentrate (11 g containing 2 g T3 and 2 g Toc) dissolved in the eluent (ethanol/water (95/5, v/v)) was continuously subjected to pilot-scale SMB separation for 13 cycles (about 15 h) (**Fig. 12**), and *B* fraction was successively withdrawn. After drying the *B* fraction, T3 (1.5 g) was obtained. The *B* fraction did not contain Toc, indicating that high purity T3 could be prepared (**Fig. 13**). Based on the pilot-scale result, a trail study for separation of rice bran T3 using an industrial-scale SMB instrument has been performed (**Fig. 14**). However, small quantity of unknown impurities seems to be contaminated into the T3 fraction. The modification of the operation parameters of the industrial-scale SMB system is therefore to be investigated. Under the new optimized condition, a large amount of high purity rice bran T3 will be prepared.

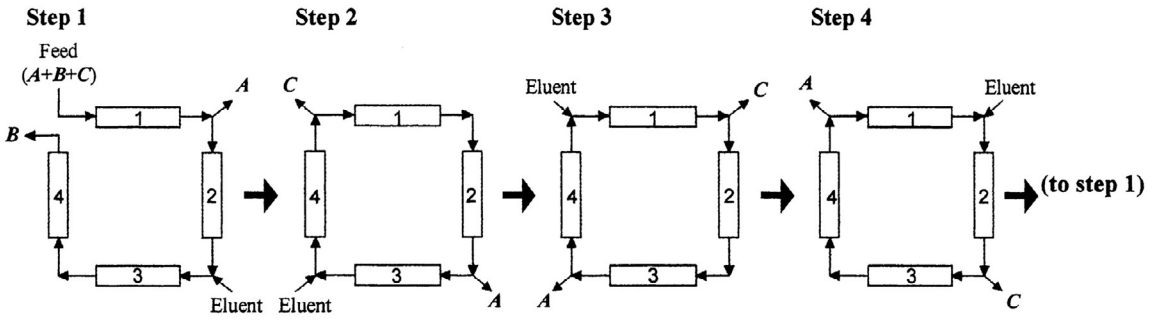


Figure 9. The process pattern of the new simulated moving-bed (SMB) chromatography for separation of three components. Where, *A* is a low affinity component, *B* is a medium affinity component, and *C* is a high affinity component.

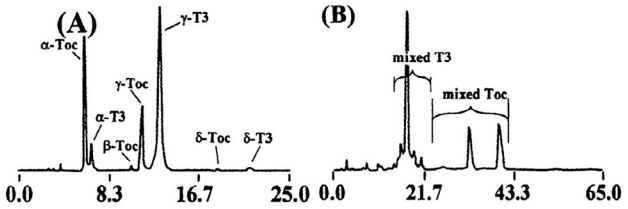


Figure 10. HPLC chromatograms of rice bran T3 and Toc. **A**. a normal-phase system (column; Zorbax Rx-SIL (4.6 × 250 mm, 5 μm, Hewlett-Packard, USA), eluent; n-hexane/ethanol (100/0.5,v/v)). **B**. a reverse-phase system (column; TSK-Gel ODS 80Ts (4.6 × 250 mm,5 μm, TOSOH, Japan), eluent; ethanol/water (80/20,v/v)).

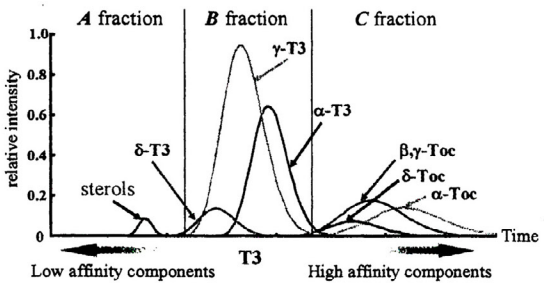


Figure 11. Computer simulation: a plot of relative intensity of each component of the rice bran concentrate. The assignment of zone separation was as *A* fraction (low affinity impurities), *B* fraction (T3 fraction), and *C* fraction (Toc and high affinity impurities).

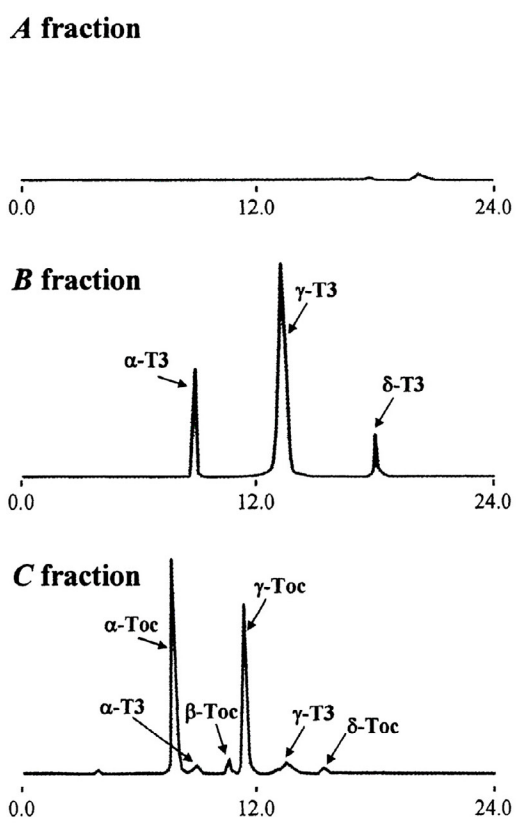


Figure 13. Chromatograms of the fractions prepared from the new SMB process.

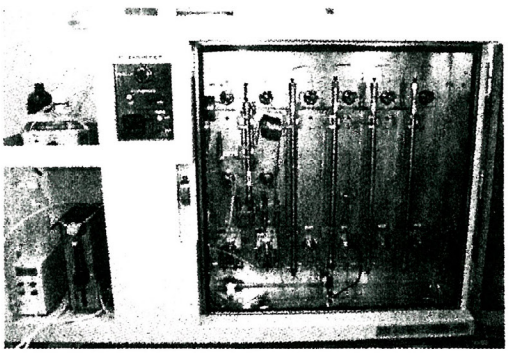


Figure 12. Illustration of a pilot-scale SMB instrument.

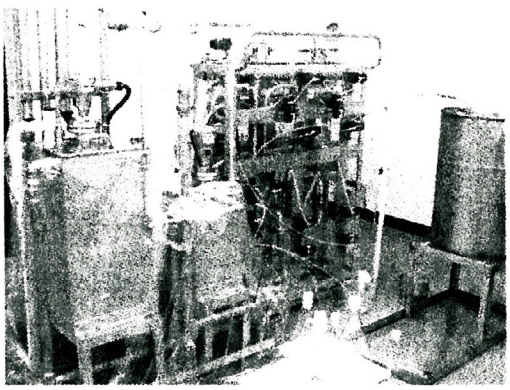


Figure 14. Illustration of an industrial-scale SMB instrument.

2-3. Enrichment of rice bran T3 in hen eggs and edible parts of chicken

Introduction Despite many functional roles of T3 being known, there have been few attempts to develop T3 food products. In this part, accumulation of T3 in eggs and edible parts of chicken (broilers) was investigated for development of T3-fortified food.

Methodology and results To observe accumulation of T3 in eggs and edible parts of chicken, 0, 2, or 4% wt rice bran scum oil (RBO, containing 1% T3) was supplemented to the feeds of laying hens and broilers. As results, T3 content in eggs was improved up to 0.6 mg/egg after 7 day of the supplementation (**Fig. 15**), while commercial eggs contained about 0.1 mg of T3/egg [10]. In the broiler experiment, the appearance of T3 was 2.5 mg/kg liver, 3.5 mg/kg breast, and 3.0 mg/kg thigh after two weeks of 2% RBO supplementation (**Table 3**). The results showed that T3 was more accumulated in eggs compared with that in broiler parts, and that T3-fortified eggs could be produced and served as one of the T3 dietary products.

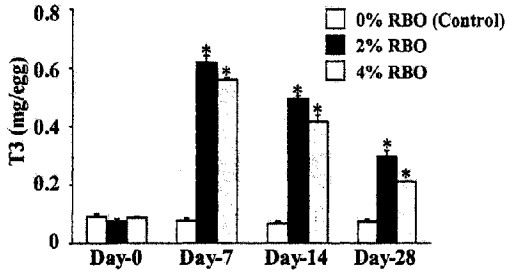


Figure 15. T3 contents in eggs collected on days 0, 7, 14 and 28. Values are Mean±SD (n = 3).

Table 3. T3 contents in liver, breast, and thigh samples of broilers after two weeks of 2% RBO supplementation.

Organ	T3 (mg/kg)	
	0% RBO (Control)	2% RBO
Liver	n.d.	2.5 ± 1.4
Breast	n.d.	3.5 ± 0.3
Thigh	n.d.	3.0 ± 0.4

Values are Mean±SD (n = 6), n.d. = non-detectable.

Conclusion

In this study, I aimed to improve T3 content in rice plants (chapter 1) and to apply rice bran T3 for nutraceutical purpose (chapter 2). A series of experiments were conducted, and following results were obtained.

Chapter 1

1-1. The developed HPLC method for determination of T3 and Toc was used in screening of 251 rice bran samples, and Milyang23 was found as the T3-rich rice variety.

1-2. The cross-fertilization of Milyang23 and the reference Koshihikari resulted in enhancement of T3 content of their F_2 progenies, in which five putative QTLs being responsible to high T3 biosynthesis were confirmed.

Chapter 2

2-1. The human daily intake of T3 was found to be 1.9-2.1 mg T3/day/person.

2-2. SMB technique was effective for large scale preparation of high purity rice bran T3 without existence of Toc.

2-3. Hen eggs had better retention of T3 than other edible broiler parts.

On the basis of this study, rice bran T3 could be better utilized (e.g., as T3 products or T3-fortified food) for promoting human health and well-being.

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International conferences

1) International Symposium: Frontiers in Rice Science – from Gene to Field, Sendai, Japan (Nov. 2006)
   Phumon Sookwong, Kiyotaka Nakagawa, Kazumasa Murata, Teruo Miyazawa
   "Production of rice plant rich in anti-angiogenic tocotrienol"
2) 10th Asian Congress of Nutrition, Taipei, Taiwan (Sep. 2007)
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   "Analysis of tocotrienol and tocopherol in rice brans"

論文審査結果要旨

米糠にはトコトリエノール（T3）とトコフェロール（Toc）が比較的多く含まれている。これらのビタミンE類には、抗酸化、血清コレステロール低下、抗腫瘍、抗血栓などの健康を維持するうえで重要な生理作用が明らかにされている。さらに最近では、T3が抗血管新生作用を有することが宮澤らにより発見され注目されている。血管新生は、癌、糖尿病性網膜症、リウマチ性関節炎などの老化性の健康障害に深く関与する。そのため、異常血管新生による組織障害を予防するための新しいアプローチとして安全性の高い食品中の血管新生抑制物質が注目されている。本研究では、我が国唯一のT3供給資源作物であるコメからT3を大量分離し応用することを目的に、T3高生産イネを作出するとともに、米糠T3を高含有する食品を開発しようとした。

はじめにT3とTocの同時迅速分析法を開発し、250種のイネ在来種の糠を分析した。その結果、通常品種（コシヒカリ）に比べ、T3をこの2倍高含有するMilyang23を有望品種として見出した。そこで、Milyang23とコシヒカリを交配してF_2イネ（133種）を得、これらF_2の中でMilyang23以上にT3を高生産するもの（32種）を選別し、それらの交配によってT3高生産形質が付与できることを明らかにした。また、QTL解析によりT3高生産に関わる染色体領域を見出した。これらの成果から、交配と選抜によりT3高生産イネの作出が可能であることを明らかにした。

T3の推奨摂取量を求めるため、これまで不明であった日本人のT3日常摂取量を、国民栄養調査と実際の機器分析により、約2mg/日/人であることを明らかにした。米油製造時の副産物であるスカム油を出発原料にして、疑似移動層クロマトグラフィーによる高純度化技術を開発し、米糠からのT3の連続製造法を確立した。これにより食品展開できる高効率な分離条件が見出され、高回収率で米油抽出残渣からの高純度T3製造を可能とした。また、T3強化食品として、T3強化卵の作出に成功した。こうしたT3高生産イネ作出技術の開発とその新食品への展開は独創的であり食品栄養学上の貢献は大きい。

よって、審査員一同は本論文提出者に対し、博士（農学）の学位を授与するに値すると判定した。